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Detection and Classification of Spontaneous Calcium Release Events in Cardiac Myocytes
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Intracellular calcium handling is a complex nonlinear process that regulates cardiac contraction and rhythm, and cardiac arrhythmias have been associated with alterations in the calcium homeostasis. The spatiotemporal behavior of anomalous calcium handling includes a rich variety of local and global spontaneous release events ranging form isolated calcium sparks to multiple calcium waves originating at the same time in different parts of a single cardiomyocyte. We have developed a novel method for automatic detection and characterization of spontaneous calcium release events from a sequence of fluorescence microscopy images. First, a mask of the cell's shape is defined using a thresholding method in order to eliminate the effects of experimental background noise. A total fluorescence signal is then obtained by averaging pixel values within the mask for each frame, which is normalized to baseline fluorescence measured in frames without activity.
The resulting signal presents a non-stationary behavior revealing the occurrence of different types of events. A wavelet-based detection method is used in order to identify independent global events in the average signal. Each event is then classified into one of three predefined types: a) single wave propagating across the cell, b) multiple simultaneous local events or c) a train of propagating waves (calcium bursts). Since the average signal does not contain local information it cannot be used to fully distinguish between these three event types. Therefore, we developed a classification method that uses a motion-tracking algorithm to identify each of the local release events contributing to a particular global event. This approach allows determining the trajectory, size, and propagation velocity of each local event and provides a reliable classification of global events. Furthermore, the method quantifies the contribution of each event type to the total calcium leakage during an experiment.

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Transitions Among Excitable, Oscillatory and Non-Excitable in Simplified Calcium Cycling Model
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Calcium (Ca) plays a central role in cardiac excitation-contraction coupling. Under normal conditions, Ca entering through the L-type Ca channel opens ryanodine receptors (RyRs), which are regulated by cytosolic ([Ca]i) and luminal Ca ([Ca]SR), and causes Ca release from the sarcoplasmic reticulum (SR). This positive feedback by Ca induced Ca release makes the Ca cycling excitable. However, as the cell goes to pathological conditions, Ca releases from the SR occurs spontaneously, leading to Ca oscillation. This transition from excitable to oscillatory can be caused by different factors, including Ca overload, RyR sensitization, SERCA uptake regulation, and structural remodeling etc. Here we use a simplified model of intracellular Ca cycling to investigate how each factor contributes to Ca cycling dynamics and shifts the system among excitable, oscillatory and non-excitable states. We find that: Increase of RyR sensitivity decreases the [Ca]SR threshold for Ca oscillation. When [Ca]SR is far from the threshold, increasing RyR sensitivity only is not enough to induce oscillation. As the system approaches [Ca]SR threshold, increasing RyR sensitivity only can induce oscillation by changing the system from excitable to oscillatory. In the oscillatory regime, increasing RyR sensitivity leads to more frequent oscillation at low [Ca]SR; However, if the RyR sensitivity becomes too low or too high, Ca oscillation is abolished. 2) Below the threshold, increase in extracellular [Ca] ([Ca]o) leads to increase in [Ca]SR and release amplitude; Above the [Ca]SR threshold, increase in [Ca]o leads to increase in oscillation frequency, decreases in oscillation amplitude and basal [Ca]SR. 3) Increase of SERCA pump increases the threshold for Ca oscillation; If SERCA is too low it cannot support Ca oscillation.

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Modeling Calcium Sparks in a Three-Dimensional Reconstruction of a Cardiac Calcium Release Unit
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A Ca2+ spark is the fundamental unit of Ca2+ release from the sarcoplasmic reticulum (SR) during excitation-contraction coupling, and the major contributor to diastolic Ca2+ leak in cardiomyocytes. The duration and magnitude of the spark is determined by the local geometry of a single Ca2+ release unit (CRU) as well as the localization and density of Ca2+ handling proteins. We have developed a detailed computational model of a single CRU situated in its native structural cellular environment. The geometry was generated using newly developed computational tools from electron microscopic tomography data, and it includes sarcoplasmic reticulum (SR), t-tubules, and mitochondria. Ca2+ diffusion is modeled both within the SR lumen and in the cytosol. The model is used to examine the effect of localization and density of the Na+ /Ca2+ exchanger (NCX) and sarco-endoplasmic reticulum Ca2+ ATPase (SERCA) pump. Our findings are: 1) including NCX in the CRU exhausts Ca2+ from the CRU during a spark significantly, 2) high SERCA density close to the CRU prolongs spark duration by preventing luminal depletion of Ca2+ ions.

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Variable RyR Cluster Morphology in Sheep Atrial Myocytes: Super Resolution Measurement and Ca2+ Release Simulation
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Conventional methods used to examine antibody labeled proteins are impeded by the resolution limits of light microscopy. We report new measurements of ryanodine receptor (RyR) clusters using the stimulated emission depletion (STED) microscopy method and correlate RyR morphology with function using a fully stochastic model of Ca2+ release. Sheep atrial myocytes were fixed and RyRs were antibody labeled and fluorescently tagged with Atto-647N. Two pulsed lasers were used for STED microscopy; a 635 nm pulsed at 80 MHz for 80ps to excite and a 780 nm laser, with a donut shaped beam was pulsed at 80MHz for 100fs for depletion. Acquired images showed ~3.5x increase in resolution compared to conventional confocal methods. After deconvolution, further improvements in both signal-to-noise ratio and resolution were observed. Gaussian fitting of the smallest detectable clusters showed a >4x improvement in resolution, allowing a typical lateral resolution of 40-65 nm. Using the protein size predicted from ultrastructure and as documented in rat ventricular myocytes, calculations of cluster size yielded a mean cluster size of 21 RyRs, with a standard deviation of 33. Over 50% of the clusters measured had a size of <7, but many small clusters were grouped in a secondary level of organisation, The influence of cluster size and geometry on Ca2+ spark generation were probed using a Monte Carlo model of Ca2+ release sites. The model contained a main release site and a smaller peripheral cluster placed at various distances from the main site to assess the affect of one cluster firing on the probability and latency of release of the associated cluster. This study highlights the usefulness of this super-resolution method to determine realistic cluster geometries and the probable consequences for the regulation of Ca2+ release.

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A Functional Analysis of GPCR and Calcium Channel Targets using Cal 520 AM Ester
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The Calcium influx assay is a preferred method for monitoring the activities of GPCR and calcium channels. Cal 520 AM is a new fluorescent calcium-sensitive dye developed for monitoring GPCR and calcium channel targets with a significantly improved signal to noise ratio and intracellular retention. Cal 520 AM ester is non-fluorescent. Once it enters the cells, the lipophilic AM blocking groups are cleaved by intracellular esterases, resulting in a negatively charged fluorescent dye that stays inside cells. When cells are stimulated with bioactive compounds, the receptor signals the release of intracellular calcium. As the dye binds to Ca2+ inside the cells, the fluorescence intensity of Cal 520 is greatly enhanced. In this study, the signal intensity and signal to background ratio of Cal 520 AM were evaluated with different receptor signaling pathways using several cell lines including HEK-293, CHO-M1 and Jurkat cells. Unlike the exisiting fluorescent calcium indicators (such as Fluo-3 AM and Fluo-4 AM) which are easily pumped out by organic-anion transporters, Cal 520 AM has much better cell retention ability in addition to its significantly higher signal to background ratio. It requires minimal amount of organic-anion

transporter inhibitors (such as probenecid) present in the assay system. Organic-anion transporter inhibitors are often toxic to cells and also interfere with the activities of bioactive compounds to be screened. In conclusion, Cal 520 AM is an improved fluorescent indicator for the measurement of intracellular calcium. The high signal-to-noise ratio and good intracellular retention properties make the Cal 520 AM a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

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Studying Calcium Signal Reshaping by Buffers Observing the Competition of Two Dyes

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Ca2+ signaling is ubiquitous across cell types. Ca2+ liberation through inositol 1,4,5-trisphosphate receptors (IP3Rs) is a key component of the Ca2+ signaling toolkit. The specificity and universality of intracellular calcium signals rely on the variety of spatio-temporal patterns that the concentration of this ion can display. It has been observed by I. Parker and collaborators that buffers can shape these patterns in different ways depending on their kinetics. In this work we study how these different effects may arise by observing IP3R-mediated Ca2+ signals using two dyes simultaneously. To this end we use a multi-spectral confocal microscope and two dyes (Fluo4 and Rhod2) that differ in their binding kinetics and emission spectra. In this way we are able to study the competition between the two dyes probing the effect of their different kinetics on the observed signals.

1579-Pos Board B349

Intercellular Calcium Waves in Vascular Smooth Muscle Cells

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Muscular arteries are able to actively modify their diameter by modulating the tone of the smooth muscle cells (SMCs) located within the arterial wall. The contractile state of the SMCs is regulated by cytosolic calcium transients and propagates as a wave over a significant distance along the vessel.

We studied the intercellular calcium wave propagation in primary cultured SMCs from rat mesenteric arteries. By using photolithography technique, two types of in vitro cells networks were developed: lines and loop patterns. We have recorded and analyzed calcium response and membrane potential variations for each cell, induced by single cell mechanical stimulus. The loop network was more efficient in transmitting the calcium signals from cell to cell. The calcium wave propagation through gap junctions was spatially limited in line patterns. Longer distance in calcium propagation was obtained in the presence of angiotensin II.

This study provides new experimental data supporting the idea that mechanical stimulation evokes a membrane potential depolarization which propagates to neighboring cells. The electrical depolarization is followed by a fast calcium entry that triggers calcium release from intracellular stores. In addition, these results suggest that increasing the expression of the gap junction protein connexin 43 by angiotensin II treatment, facilitates longer distance propagation of calcium waves in primary cultured SMCs.

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Sarcoplasmic Reticulum Calcium Leak in Normal and Dystrophic Skeletal Muscle

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Under resting conditions, external calcium is known to enter skeletal muscle cells while calcium stored in the sarcoplasmic reticulum (SR) leaks into the cytosol. The nature of the pathways involved in the resting sarcolemmal calcium entry and in the SR calcium leak are still debated but several lines of evidence suggest that an up-regulation of these calcium fluxes occurs in Duchenne Muscular Dystrophy (DMD). We investigated here SR calcium permeation at resting potential and in response to depolarization in voltage-controlled skeletal muscle fibers from control and *mdx* mice, the murine model of DMD. Using the cytosolic calcium dye Fura2, we first demonstrated that the rate of calcium increase induced by CPA (cyclopiazonic acid) inhibition of SR Ca^{2+}-ATPases at resting potential is significantly higher in *mdx* fibers suggesting an elevated SR passive calcium leak. However, in these experiments, sarcolemmal calcium influx may contribute to the CPA-induced calcium increase and another series of experiments indicated that CPA-induced SR calcium leak was deeply modified in the absence of external calcium. Fibers were then loaded with the low affinity calcium dye Fluo5N-AM and dialyzed with 50 mM EGTA to measure intraluminal SR calcium changes. Depolarization pulses evoked voltage-dependent Fluo5N fluorescence decreases followed by a recovery phase which was inhibited by CPA, demonstrating that Fluo5N actually reports intraluminal SR calcium changes. Voltage-dependence and magnitude of depolarization-induced SR calcium depletion were found to be unchanged in *mdx* fibers but the rate of the recovery phase that followed depletion was found to be faster, suggesting a higher SR calcium reuptake capacity in *mdx* fibers. Finally, CPA-induced SR calcium leak at −80 mV was found to be significantly higher in *mdx* fibers. The elevated SR passive calcium leak may participate to the muscle degenerative process in *mdx* muscle.

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Altered Skeletal Muscle Excitation Contraction Coupling in Dysferlinopathy

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Limb Girdle Muscular Dystrophy Type 2B (LGMD2B) and Miyoshi Myopathy (MM) are caused by mutations in the dysferlin gene, but the role of dysferlin in healthy muscle and the changes that occur when it is mutated or absent are poorly understood. Previous work supported a role for dysferlin in sarcolemmal repair following laser wounding or other in vitro injuries. We study the response of A/J mice, which lack dysferlin, to injury by large-strain lengthening contractions in vivo. We find that dysferlin promotes normal recovery from this physiological injury but is not necessary for sarcolemmal repair. Consistent with this, immunofluorescence microscopic studies of healthy muscle, that we fixed and treated in a hot, mildly acidic solution to expose dysferlin's epitopes, show that dysferlin is primarily in transverse tubules (TT), not the sarcolemma as previously reported. Furthermore, TT are disrupted when skeletal muscle is injured physiologically, and disruption is much more extensive in A/J muscles than in controls. Studies of FDB myofibers in tissue culture also demonstrate the presence of dysferlin in TT. Brief exposure of control myofibers to hypoosmotic solutions damages TT, in a process dependent upon extracellular Ca2+. As in vivo, A/J myofibers are more extensively damaged by osmotic shock than controls; they are indistinguishable from controls when shocked in Ca2+-free medium, however. Thus Ca2+ may promote damage, rather than participate in dysferlin-dependent membrane repair, as previously reported. Our results suggest that the changes in TT following injury in vivo and in vitro are similar, that they require extracellular Ca2+, and that they are much more pronounced when dysferlin is absent. We propose that dysferlin is essential for the integrity of the TT of skeletal muscle, in maintaining this integrity during contraction and relaxation, and in repairing damaged TT following injury.

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Caffeine Treatment and Depolarization Alter the Spatial and Temporal Characteristics of Calcium Sparks on Intact Amphibian Skeletal Muscle

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Calcium sparks were recorded on intact skeletal muscle fibers of the frog using high time resolution confocal microscopy (x-y scan: 30 Hz, line-scan: 60 kHz). Sparks were elicited by 1 mmol/l caffeine or subthreshold depolarization to different membrane potentials (data presented for −60 mV). Both treatments increased the frequency of sparks and altered their morphology. Images were analyzed by custom-made computer programs. Both the amplitude (in $\Delta F/F_0$; 0.49 ± 0.025 vs. 0.29 ± 0.001; n = 22426 vs. 23714; mean ± SEM, $p<0.05$) and the full width at half maximum (FWHM, in μm; parallel with fiber axis: 2.33 ± 0.002 vs. 2.21 ± 0.005; perpendicular to fiber axis: 2.07 ± 0.003 vs. 1.88 ± 0.004) of sparks was significantly greater after caffeine treatment than on depolarized cells. On x-y scans 25.8% (caffeine) and 16.4% (depolarization) of detected sparks overlapped with another one on the previous frame. Center of signal mass of overlapping sparks travelled shorter distances between consecutive frames after caffeine treatment then after depolarization (in μm; 1.80 ± 0.017 vs.